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Effects of hormonal stimulation on the concentration and quality of excreted spermatozoa in the critically endangered Panamanian golden frog (*Atelopus zeteki*)

Gina Della Togna [a], Vance L. Trudeau [b], Brian Gratwicke [a], Matthew Evans [c], Lauren Augustine [c], Han Chia [a], Edward J. Bronikowski [c], James B. Murphy [c], Pierre Comizzoli [a, *]

[a] Smithsonian Conservation Biology Institute, Smithsonian National Zoological Park, 3001 Connecticut Ave., NW, Washington, DC, 20008, USA
[b] Department of Biology, University of Ottawa, 30 Marie Curie St., Ottawa, ON, K1N 6N5, Canada
[c] Center for Animal Care Sciences, Smithsonian National Zoological Park, 3001 Connecticut Ave., NW, Washington, DC, 20008, USA

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ABSTRACT

Knowledge of basic gamete biology is critical to better protect and propagate endangered amphibian species and also to develop reproductive technologies combined with germplasm cryopreservation. The objectives of the study were to test different hormonal stimulations and then characterize the quantity and quality of Panamanian golden frog *(Atelopus zeteki)* spermatozoa. Following intraperitoneal injection of the gonadotropin-releasing hormone agonist (des-Gly^{10}, D-Ala^{6}, Pro-NHEt^{9}-GnRH 1, 2 or 4 μg/g of body weight), human chorionic gonadotropin (hCG; 5 or 10 IU/gbw), or Amphiplex™ (0.4 μg/gbw GnRH-A + 10 μg/gbw metoclopramide hydrochloride), spermic urine samples from 29 males were collected at different time points (from 0.5 to 24 h post-injection) to analyze the concentration, motility, and morphology of the spermatozoa. Peak of sperm concentration was observed at 3.5 h post injection for all hormonal treatments. Amphiplex™ led to the highest sperm concentrations ($4.45 \pm 0.07 \times 10^6$ cells/mL) followed by 4 μg/gbw GnRH-A ($2.65 \pm 0.21 \times 10^6$ cells/mL). Other stimulation protocols and doses induced sperm production, but at lower levels (ranging from 1.34 to 1.70×10^6 cells/mL). More than 60% of spermatozoa were motile following all treatments but the highest motility (>90%) was obtained from the 10 IU/gbw hCG treatment. Spermic urine samples obtained with all hormone treatments had higher pH (ranging from 7.1 to 7.8) than the urine alone (6.7–6.8). Spermatozoa were filiform and elongated with an apical acrosome, a mitochondrial sheath, a small midpiece and a long tail with an undulating membrane. More than 80% of cells were morphologically normal and 50–70% had intact DNA. These sperm characteristics were not influenced by hormonal treatments. This first comprehensive characterization of sperm samples following optimized hormonal stimulations in *A. zeteki* lays the foundation for more fundamental studies, reproductive technologies, and future preservation strategies.

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1. Introduction

Amphibian populations around the world are experiencing drastic declines with 31% considered being threatened with extinction (http://www.iucnredlist.org). The genus *Atelopus* represents the largest group in the Bufonidae family (Anuran order) found in 11 countries in Central and South America [1,2]. About 80% of *Atelopus* species now are threatened with extinction and 56% of those have probably disappeared from their known habitats [1,3] mainly due to the infectious fungal disease chytridiomycosis, climate change, pollution, habitat destruction and the pet trade [1,4]. The Panamanian golden frog (*Atelopus zeteki*) is an endemic species from Panama that has not been observed in the wild since 2009, is listed as Critically Endangered (CR) by the IUCN, and is protected under Panamanian law [5,6]. Fortunately, a healthy captive assurance population populations of *A. zeteki*, critical for the preservation of remaining populations, is bred in U.S. zoos and aquaria [7,8].

Improved understanding of reproductive physiology will help

* Corresponding author.
E-mail address: comizzolip@si.edu (P. Comizzoli).

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successful breeding programs to sustain populations of genetically diverse individuals [9–11]. However, successful unassisted breeding of amphibians in captivity can be difficult because of issues related to poor genetic diversity, unsuccessful or asynchronous spawning, absence of male breeding behavior, dystocia, low survival rates of eggs, embryos and juveniles, as well as diseases [10,12]. Exogenous hormone stimulations to collect spermatozoa and oocytes for artificial fertilization, or for refrigerated or cryopreserved storage, therefore represent a great option to help sustain healthy, genetically viable captive populations over the long term [9].

Males amphibians excrete spermic urine following hormonal stimulations but results vary among species [9,13,14]. Human chorionic gonadotropin (hCG) has been widely used in numerous amphibian species including *Xenopus, Bufo* and *Litoria* to collect spermic urine [15–18]. Additionally, gonadotropin-releasing hormone (GnRH) agonists have been used alone or in combination with dopamine antagonists such as metoclopramide, domperidone or pimozide [11,19,20]. Fresh or frozen–thawed spermic urine has been used to fertilize oocytes in Anurans [9]. However, no reports on sperm collection after hormonal stimulation are available in *Atelopus* sp.

In addition to sperm concentration, essential traits such as motility, cell morphology, osmolality and pH of the spermic urine (even before the fertilizing ability) have to be assessed for an accurate evaluation of the sperm sample [21]. Sperm motility is usually considered an indicator of fertilization success, frequently associated with the type of fertilization and the spawning environment of a given species [22–25]. In anurans as in fish, sperm motility is triggered by osmotic changes in the extracellular environment [24,26,27]. Cells are immotile inside the testicular tissue that has an osmolality of ~300 mOsm/kg [12,25,27]. Hypotonic environments such as urine or fresh water then activate sperm in anurans and fresh water fish. Sperm motility is also affected by the pH of the extracellular medium with a neutral pH providing the best conditions for motility as observed in *X. laevis* [25]. Sperm morphology and DNA integrity are additional important traits to consider when evaluating sperm quality since both are essential for successful fertilization and embryo development [28,29]. Lastly, the only study on the reproductive ecology of *A. zeteki* reports that the species breeds from late November to January, during the start of the dry season [30]. However, no data are available on the seasonality of the spermic urine production under captive conditions.

Based on the current state of knowledge in *A. zeteki*, the objective of this study was to characterize the effect of the repeated hormonal stimulations throughout the year on sperm concentration and quality.

2. Materials and methods

2.1. Male husbandry and identification

Adult males *Atelopus zeteki* (>two year old, average weight of 3.81 ± 0.41 g) were obtained from the Species Survival Plan (SSP) 'Mata Ahogado' managed sub-population at the Maryland Zoo in Baltimore, MD. Animal Use Protocols were submitted for the Institutional Animal Care and Use Committee (IACUC) approval from the University of Maryland, the Smithsonian's National Zoological Park (SNZP) in Washington, D. C., and Maryland Zoo in Baltimore, MD. After obtaining IACUC approvals, the frogs were transferred from the Maryland Zoo to the SNZP and were placed under quarantine for 30 days. For identification purposes, four photographs of each frog were taken to distinguish males utilizing their spot patterns and an alpha-numeric code was assigned to each male.

Up to 5 frogs were housed at 20–22 °C in each of 6 tanks (Exo-Terra Terrarium, 24″×18″×24″). Tanks were furnished with live potted plants and live moss as substrate, misted with reverse-osmosis-filtered water once every hour for 2 min and drained through a bulkhead. Tanks were lit on a 12-h cycle with Zoomed10.0 T5 high output UV-B bulbs to avoid D_3 hypovitaminosis and help with proper bone growth [31]. Frogs were fed *ad libitum* four times per week with live one-week-old crickets dusted with a calcium carbonate powder containing vitamin D_3, wingless fruit flies (*Drosphila melanogaster*) or bean beetles (*Callosobruchus* sp.) on a rotating basis.

2.2. Hormone stimulation

Treatment dose was chosen based on preliminary dose-range experiments with *A. zeteki* and previous successful induction of spawning in other species [9,11,20,21,32]. Hormonal stimulations were administered via intraperitoneal injection (ip) using Terumo Thinpro insulin syringes (30 ga x 3/8″). The different hormonal stimulation were as follows: 1) 1 μg/g of body weight (gbw) of the gonadotropin-releasing hormone agonist des-Gly^{10}, D-Ala^{6}, Pro-NHEt^{9}-GnRH (GnRH-A; Sigma-Aldrich Corporation, St. Louis, MO, USA), 2) 2 μg/gbw GnRH-A 3) 4 μg/gbw GnRH-A, 4) 5 IU/gbw hCG (CG5 Chorionic gonadotropin human; Sigma-Aldrich Corporation), 5) 10 IU/gbw hCG, or 6) Amphiplex™ - a cocktail of 0.4 μg GnRH-A plus10 μg/gbw metoclopramide hydrochloride (Sigma-Aldrich Corporation) named after the combination of the words 'amphibian' and 'amplexus' [11,20]. All hormones were dissolved in Amphibian Ringer Solution (ARS; Carolina Biological Supply Company, Burlington, NC, USA) and control males received 10 μl/gbw (ip) of ARS or no injection at all.

2.3. Sperm collection

Session of sperm collections were scheduled once a week. For each session, six males were selected to be induced with one of six hormone treatments with two additional individuals as controls. After hormonal and control injections, males were placed inside ventilated plastic containers with water soaked paper towel on the bottom and covered from light to reduce stress. Spermic urine was collected by gently inserting a small catheter (0.6 mm I.D. x 1 mm O.D., Micro Medical Tubing, 85 Durometer Vinyl, Scientific Commodities Inc., Lake Havasu City, AZ, USA) in the cloacae (Fig. 1) [33]. Sperm collections were carried out at seven different time points post injection: 0.5, 1.5, 2.5, 3.5, 4.5, 5.5 and 24 h. Testicular spermatozoa from eleven euthanized males from another study also were collected after dissection and maceration of the testes in 500 μL ARS, followed by short-term storage at 4 °C until samples

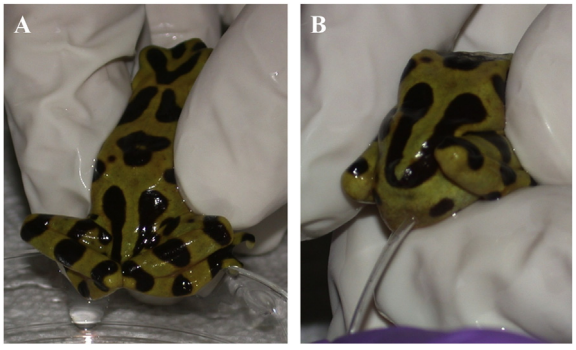

Fig. 1. Handling of Panamanian golden frog (A) and catheter positioning for the collection of spermic urine (B).

were analyzed.

2.4. Sperm processing and analysis

Volume of sample recovered from the catheter was measured with a 200-μL micropipettor. Sperm concentration was assessed by hemocytometery with 10 μL spermic urine being added to the chamber. The osmolality and pH of the spermic urine were obtained using a Vapro Osmometer 5520 (Wescor, Inc, South Logan, Utah, USA) and pH indicator strips pH 5.0–10.0, EMD Millipore, Billerica, MA, USA), respectively. Percentage of sperm motility was determined by counting all the cells with flagellar movement from a total of 100 cells under an Olympus BX41 microscope at X 400 magnification. Percentage Forward progressive motility (FPM) was obtained by counting all the cells with forward motility relative to 100 cells that were expressing any flagellar movement.

2.5. Sperm morphology analysis

Morphology was assessed by fixing 10 μL spermic urine in 80 μL 4% paraformaldehyde solution in PBS. Fixed cell then were stained with Coomassie Blue (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) and mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA). Sperm morphology was evaluated at X 1000 magnification under an Olympus BX41 microscope. In a first assessment, a maximum of 100 cells were classified as normal if every morphological parameter of the cell was intact (acrosome, head, mitochondrial sheath and tail) and as abnormal if at least one of the parameters was not intact. In a second reading, a maximum of 100 cells per sample were evaluated in more details for acrosomal integrity [normal (intact) and abnormal (not intact, missing)], head [normal (intact) and abnormal (bent, broken)] and tail [normal (intact) and abnormal (coiled, with cytoplasmic droplet or broken)]. Images of the cells were acquired with a SPOT RT3™ Color Slider microscope camera and the SPOT Advanced Software (SPOT Imaging Solutions, Sterling Heights, MI, USA). Measurements of the head and tail were obtained from 100 cells from different individuals with the SPOT Advanced Software segmented line measuring tool.

Testicular spermatozoa from eleven euthanized males from another study were also used as a comparison for the morphology assessment (as described above).

For DNA staining, an aliquot of 10 μL of cells previously fixed in 4% parafolmaldehyde was stained with 3 μL of either Hoechst (excitation/emission of 338/505 nm, 50 mg/mL) or PI (absorption 493 nm, 50 mg/mL) and analyzed under a fluorescence Olympus BX41 microscope (X400). For the mitochondrial sheath staining, 1 μL of 100 nM MitoTracker® Green FM (Life Technologies, Grand Island, NY, USA), excitation/emission of 490/516 nm, was added to 10 μL of fresh spermatozoa. After 15 min, cells were fixed in 4% parafolmaldehyde and analyzed under fluorescence Olympus BX41 microscope (X1000).

2.6. Assessment of sperm DNA integrity

Sperm DNA fragmentation was determined using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique (DeadEnd™ Colorimetric TUNEL System, Promega Corporation, Madison, WI, USA), with an *in situ* cell detection kit, following the manufacturer's instructions. Ejaculated spermatozoa used in this assay were previously fixed in 4% paraformaldehyde and kept at 4 °C until analyzed. Slides were prepared by smearing the samples and letting them air-dry. Slides were then gently rinsed twice with PBS for 5 min before the cells were permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Slides were again gently rinsed twice with PBS for 5 min followed by incubation with the equilibration buffer for 10 min at room temperature. Then, 100 μL of previously prepared rTdT reaction mix were added to the slides. After covering with plastic coverslips, slides were incubated at 37 °C for 60 min in a humidified chamber. The reaction was terminated by removing the coverslips and adding 200 μL of 2X SCC to each slide using a pipette, incubating them for 15 min at room temperature. Slides were rinsed twice with PBS for 5 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 5 min and then the slides were again rinsed twice with PBS for 5 min followed by incubation with 200 μL Streptavidin Horseradish Peroxidase for 30 min. Slides were rinsed twice with PBS for 5 min and developed with 3,3′-diaminobenzidine before being mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA) and analyzed at X 1000 magnification under an Olympus BX41 microscope. One hundred cells were counted per slide and classified as intact (no staining) or fragmented (dark brown staining). Testicular spermatozoa from 11 euthanized males from another study were used as a control for the TUNEL assay as well.

2.7. Seasonality

To assess possible seasonal variations in *A. zeteki* sperm concentration, spermic urine osmolality and pH after hormonal stimulation, we divided the year in four blocks (seasons) of three months each, starting with season 1 from January to March, season 2 from April to June, season 3 from July to September and season 4 from October to December.

2.8. Statistical analysis

For each hormonal treatment, sperm concentration peaks (time points with the highest sperm concentrations) were determined using a cutoff value that was calculated by averaging the total sperm concentration of all time points for each treatment plus one time the standard deviation (SD). The time points with sperm concentrations equal or above the cutoff were removed (as part of the peak) and a new cutoff value was calculated with the remaining time points. This process was repeated until there was no time point left with a sperm concentration equal or higher than the cutoff. All values equal or higher than the cutoffs were considered the most elevated sperm concentrations and part of the peak of sperm production for each hormone treatment [34] (Table 1).

All statistics were carried out utilizing the GraphPad Prism 6.05 software (GraphPad Software, Inc., La Jolla, CA, USA). All data were examined to determine normality and homogeneity of variance using the Brown-Forsythe test. A one-way ANOVA and Tukey's post-hoc test for multiple comparisons were used to determine differences among the treatments. For the effect of treatments on morphology of cells, we tested for differences using one-Way ANOVA followed by Dunn's and Tukey's test for multiple comparisons, or the Kruskal-Wallis test when variances were not homogeneous. Means were considered statistically different if $P < 0.05$. A Spearman correlation test was used to examine the relationship between osmolality and pH, FPM, and between pH and osmolality, Motility and FPM.

3. Results

3.1. Effect of exogenous hormone treatments on the sperm excretion pattern

Compared to both controls, only hormonal injections led to the

Table 1
Calculation of sperm concentration peaks by hormonal treatment. The symbol (/) indicates no more time points that were selected for a specific cutoff.

Treatment	Overall concentration ($\times 10^6$ cells/mL)	Cutoff 1 (cells/mL)	Peak time point selected	Cutoff 2 (cells/mL)	Peak time point selected	Cutoff 3 (cells/mL)	Peak time point selected	Cutoff 4 (cells/mL)	Peak time point selected	Total # of time points/peak
1 μg/gbw GnRH −A	0.92 ± 0.76	1.68×10^6	1	1.38×10^6	2	0.50×10^6	0	/	/	3
2 μg/gbw GnRH-A	1.02 ± 0.66	1.68×10^6	2	1.26×10^6	1	0.99×10^6	1	0.68×10^6	0	4
4 μg/gbw GnRH-A	1.94 ± 0.75	2.69×10^6	1	2.45×10^6	1	2.22×10^6	0	/	/	2
5 IU/gbw hCG	1.0 ± 0.65	1.65×10^6	1	1.46×10^6	1	1.31×10^6	1	1.10×10^6	0	3
10 IU/gbw hCG	1.06 ± 0.68	1.74×10^6	1	1.01×10^6	3	0.67×10^6	0	/	/	4
Amphiplex™ (0.4 μg/gbw GnRH-A + 10 μg/gbw metoclopramide)	2.23 ± 1.81	4.04×10^6	2	2.54×10^6	0	/	0	/	/	2

production of spermic urine samples (Fig. 2A–F). Sperm excretion was obtained as early as 0.5 h post-injection with all GnRH-A treatments, 10 IU/gbw hCG and Amphiplex™ (Fig. 2A–C, 2E–F), while no production of sperm was obtained at this time point after treatment with 5 IU/gbw hCG (Fig. 2D). However, for all treatments spermatozoa were mainly collected starting at 1.5 h and up to 24 h post-injection (Fig. 2A–F). Following hormonal stimulations, sperm concentrations in samples ranged from 0.0 to 8.20 $\times$ 10^6 cells/mL (Table 2). The peak of excretion lasted longer with 2 μg/gbw GnRH-A, followed by 10 IU/gbw hCG and was shorter for 4 μg/gbw GnRH-A and Amphiplex™ treatments (Table 2). Interestingly, all treatments had 3.5 h post-injection as the common time point for peak sperm excretion (Table 2). When comparing the samples obtained during the peaks only, 4 μg/gbw GnRH-A and Amphiplex™ led to higher sperm concentrations (2.65 ± 0.21 $\times$ 10^6 and 4.45 ± 0.07 $\times$ 10^6 cells/mL, respectively; $P < 0.05$) than the other hormonal stimulations (Table 2; Fig. 3A).

From 0.5 to 24 h post-injection as well as during the excretion peaks, the volume (range, 10–270 μL) and the osmolality (range, 56–130 mOsm/kg) varied at each time point but were not affected ($P > 0.05$) by the various hormonal treatments. However, the pH was consistently higher ($P > 0.05$) after hormonal stimulation than in the controls. For the spermic urine, pH was lower after treatment with 4 μg/gbw GnRH-A (7.15 ± 0.07) than 5 IU/gbw hCG (7.86 ± 0.05; $P < 0.05$) while no differences in pH were observed with the other treatments (Fig. 3B).

For the most successful hormonal treatments (4 μg/gbw GnRH-A, 10 IU/gbw hCG, and Amphiplex™), sperm concentrations were not significantly affected by the seasons of the year (Fig. 4). The concentration of sperm in urine obtained from males injected with

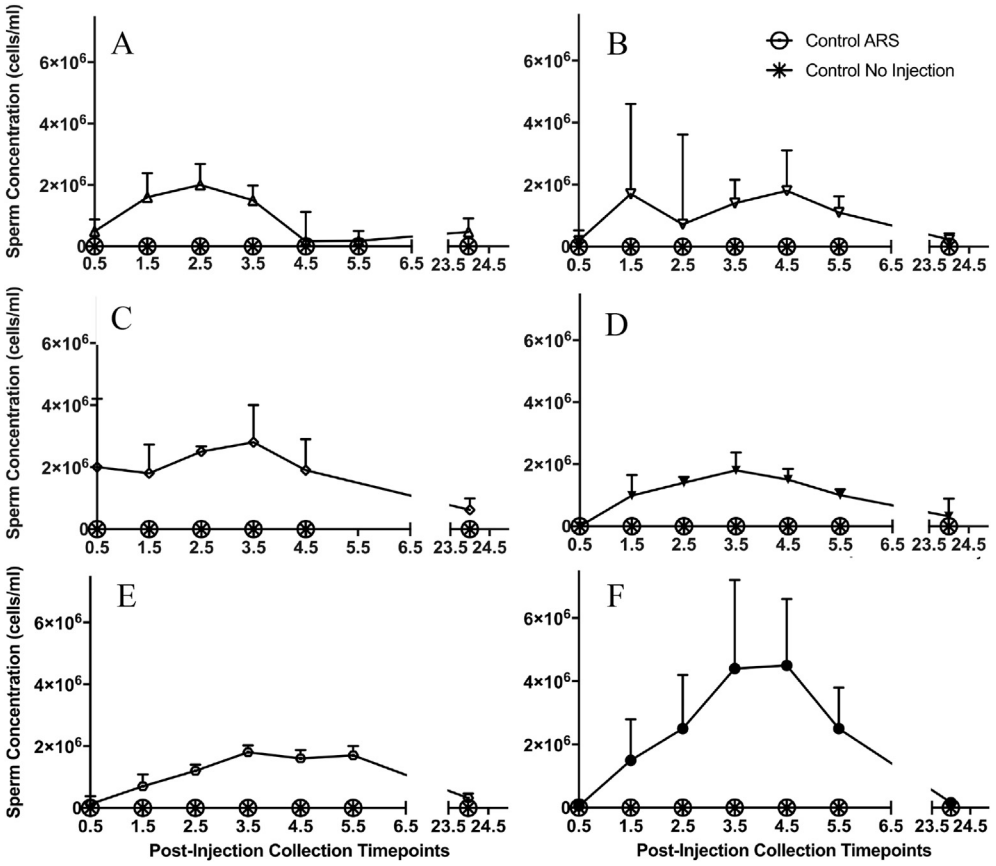

Fig. 2. Sperm concentration at each post-injection time point for each hormone treatment (n = 24 males) and two controls (n = 12 males): 1 μg/gbw GnRH-A (A), 2 μg/gbw GnRH-A (B), 4 μg/gbw GnRH-A (C), 5 IU/gbw hCG (D), 10 IU/gbw hCG (E), Amphiplex™ (0.4 μg/gbw GnRH-A+ 10 μg/gbw metoclopramide; F). Values are expressed as mean ± SD.

Table 2
Characteristics of sperm concentrations according to the hormonal stimulation.

Treatment	Overall concentration ($\times 10^6$ cells/mL)	Range ($\times 10^6$ cells/mL)	Peak of concentration (h post-injection)	Average concentration at peak ($\times 10^6$ cells/mL)
1 μg/gbw GnRH −A	0.92 ± 0.76	0.010–3.72	1.5 to 3.5	1.70 ± 0.26
2 μg/gbw GnRH-A	1.02 ± 0.66	0.007–8.20	1.5 to 5.5	1.34 ± 0.39
4 μg/gbw GnRH-A	1.94 ± 0.75	0.031–5.30	2.5 to 3.5	2.65 ± 0.21
5 IU/gbw hCG	1.0 ± 0.65	0.000–2.80	2.5 to 4.5	1.56 ± 0.20
10 IU/gbw hCG	1.06 ± 0.68	0.010–2.26	2.5 to 5.5	1.57 ± 0.26
Amphiplex™ (0.4 μg/gbw GnRH-A+ 10 μg/gbw metoclopramide)	2.23 ± 1.81	0.020–7.98	3.5 to 4.5	4.45 ± 0.07

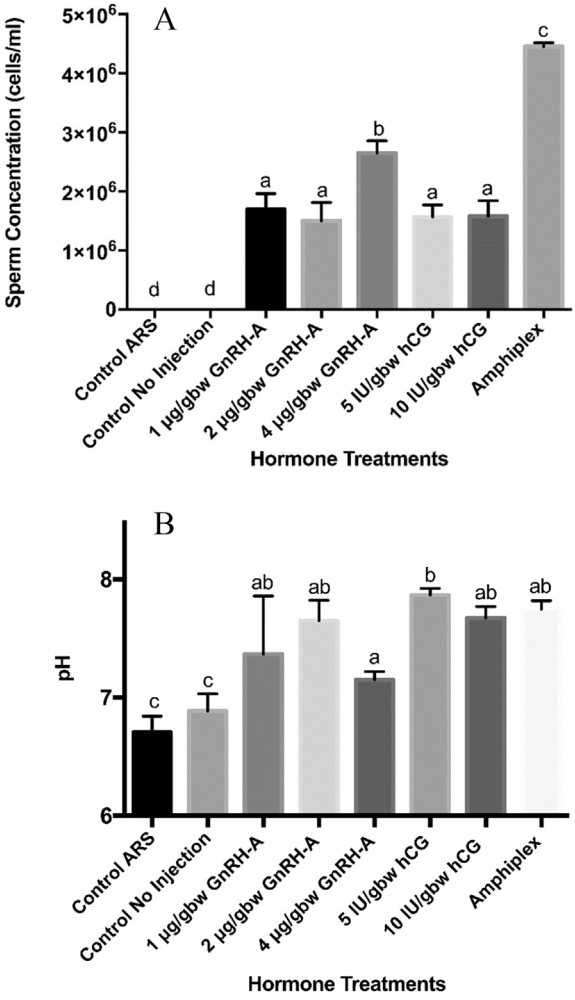

Fig. 3. Sperm concentrations (A) and spermic urine pH (B) measured during peaks of excretion after different hormonal treatments (n = 24 males) and two controls (n = 12 males). Values are expressed as mean ± SD. Bars with different letters are statistically different (P < 0.05).

Amphiplex™ remained elevated throughout the entire year compared to the other two treatments (Fig. 4).

Percentages of sperm motility during the peak of excretion were all above 86% (range, 86.7–100%) regardless of the hormonal treatment. Lowest percentages of motility (P < 0.05) were observed with 4 μg/gbw GnRH-A (87.2 ± 0.8%) while highest sperm motility (P < 0.05) was obtained with Amphiplex™ (99.1 ± 1.3%; Fig. 5A). Regarding the sperm motility pattern, lowest percentages of spermatozoa with forward progressive motility were observed after the stimulation with 2 μg/gbw GnRH-A (68.9 ± 7.3%) while the highest was obtained with 10 IU/gbw hCG (95.4 ± 2.1%; P < 0.05; Fig. 5B).

3.2. Effects of exogenous hormonal stimulation on the sperm morphology and DNA integrity

Spermatozoa of *A. zeteki* have an elongated shape (Figs. 6A and 7). The anterior part of the head exhibits a pointy acrosome (Fig. 6Aa) at the tip of a long head (24.5 ± 2.9 μm including the acrosome; Fig. 6Ab) containing the compacted chromatin (Fig. 7F and G). The mitochondrial sheath is a cytoplasmic droplet like structure positive to Mito Tracker Green FM staining that is located towards the center of the head (Figs. 6Ac, 7B and 7D). A small midpiece at the base of the head (Fig. 6Ad) is connecting the head to the sperm tail (8.0 ± 5.6 μm length) and exhibits two filaments joined together by an undulating membrane (Fig. 6Ae).

Abnormal morphology including missing acrosomes, missing mitochondrial sheaths or coiled tails were observed in all samples (Fig. 6B–E), but the vast majority had normal morphology, regardless of the hormonal treatment (Fig. 8). Percentages of normal sperm heads and acrosomes were similar between hormonal stimulations and did not differ from the testicular sperm control (Fig. 8A and B). Multiple abnormal morphologies including missing acrosomes, missing mitochondrial sheath or coiled tail were observed in the samples (Fig. 6B–E). The only difference observed was the lower percentage of normal tail in testicular samples (P < 0.05; Fig. 8C). Spermatozoa collected after the most effective hormonal stimulations (4 μg/gbw GnRH-A, 10 IU/gbw hCG and Amphiplex™) were also assessed for their DNA integrity. Percentages of intact DNA ranged from 12.0 to 96.7% and statistically did not differ between treatments or the testicular sperm control (P > 0.05; Fig. 8D).

4. Discussion

For the first time, sperm samples from captive *A. zeteki* were successfully collected throughout the entire year using hormonal stimulations. The quantity and quality of the gametes was affected by the various hormone preparations with GnRH-A plus metoclopramide resulting in the best collections.

In this study the effectiveness of the different hormone treatments in inducing spermiation were quite varied, as observed in other anurans [35]. Successful induction using GnRH-A alone was similar to responses observed in other Bufonids [21,32,36]. In our conditions, GnRH-A induced the production of significantly higher concentrations of sperm than hCG as observed in species like the Corroboree frog *Pseudophryne corroboree* [37] but not in other Bufonids. For example, *B. fowleri* responding better to hCG than GnRH [17]. Time-dependent increases in sperm concentration have also been observed using hCG and GnRH-A in some bufonid species [18,38]. For instance, a study in *Anaxyrus americanus* showed that hCG stimulation produced a peak between 3 and 9 h post-injection and no clear peak of sperm production between 3 and 24 h post-injection of GnRH-A [18]. This is different from what we observed in *A. zeteki* where distinct sperm concentration peaks were

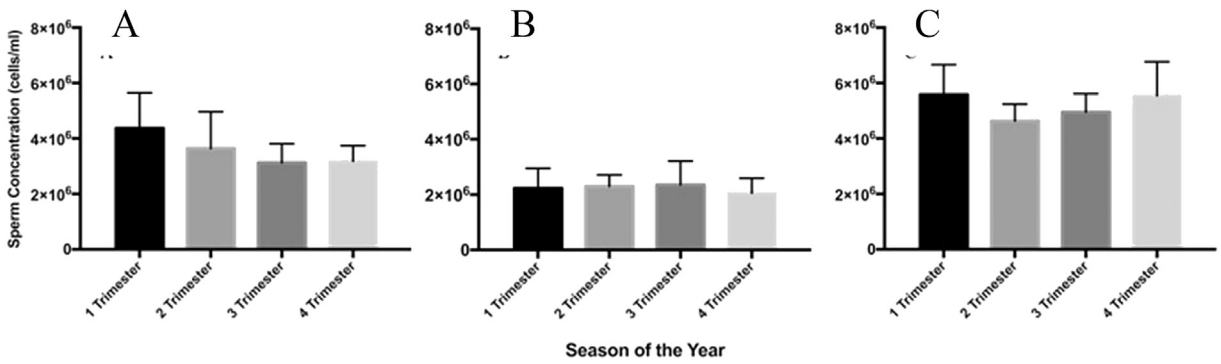

Fig. 4. Sperm concentration during the 4 seasons of the year after treatment with 4 μg/gbw GnRH-A (A), 10 IU/gbw hCG (B) and Amphiplex™ (C). Values are expressed as mean ± SD.

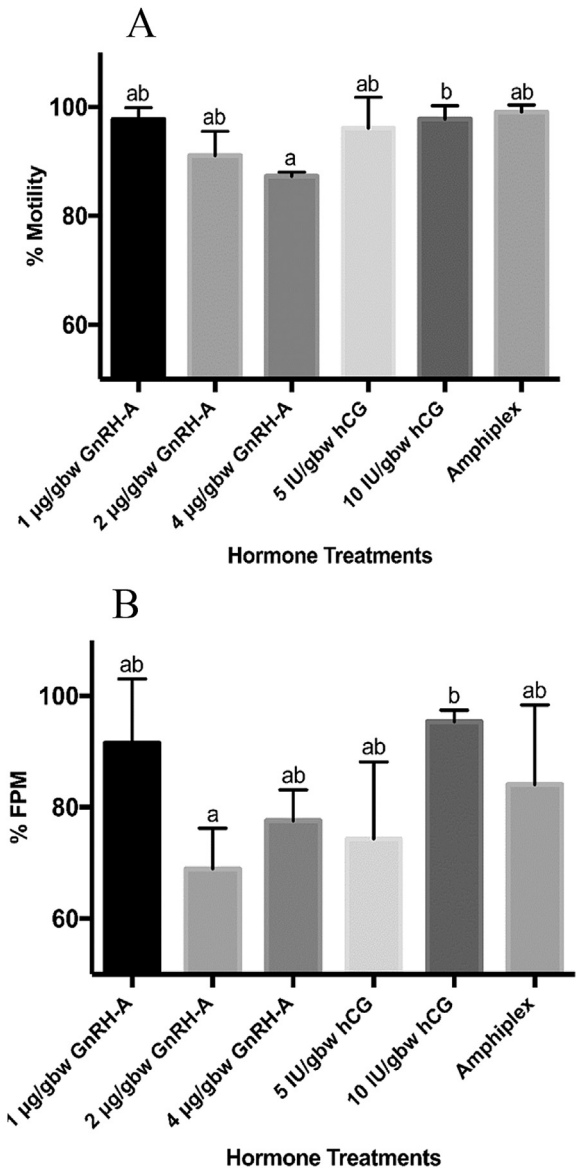

Fig. 5. Percentage sperm motility (A) and sperm forward progressive motility (FPM; B) measured during the peak of excretion after different hormone treatments (n = 24 males). Values are expressed as mean ± SD. Bars with different letters are statistically different ($P < 0.05$).

obtained after stimulation with GnRH-A as well which is consistent with studies in *Rana temporaria* [38]. Frogs treated with Amphiplex™ reached a sperm production peak at 3.5 h post-injection, with 4 μg/gbw GnRH-A eliciting a more rapid response. In comparison, Amphiplex™ induced the production of signifantly higher concentrations of spermatozoa. This is interesting because Amphiplex™ contains only 0.4 μg/gbw GnRH-A, which is 10 times less than the 4 μg/gbw GnRH-A, and this suggests that metoclopramide likely potentiated the effect of the lower GnRH-A

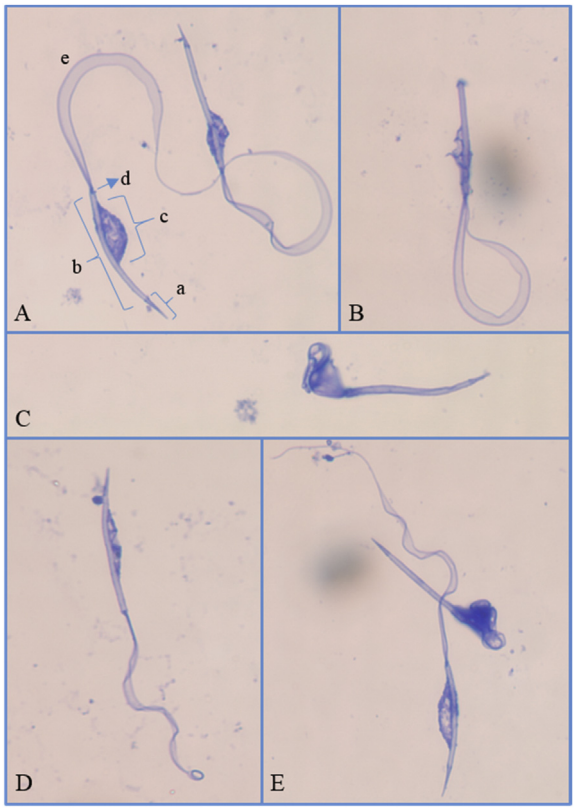

Fig. 6. Micrographs of Panamanian golden frog spermatozoa stained with Coomassie Blue (X 1000 magnification). Normal spermatozoa (A) with acrosome (a), head (b), mitochondrial sheath. (c), midpiece (d) and tail (e). Abnormal spermatozoon (B) missing the apical acrosome and with a broken sheath. Abnormal spermatozoon (C) with a coiled tail and no mitochondrial sheath. Abnormal spermatozoon (D) with an abnormal acrosome and coiled tail at the posterior end. Abnormal spermatozoon (E) with a broken tail or with a coiled tail and no sheath. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

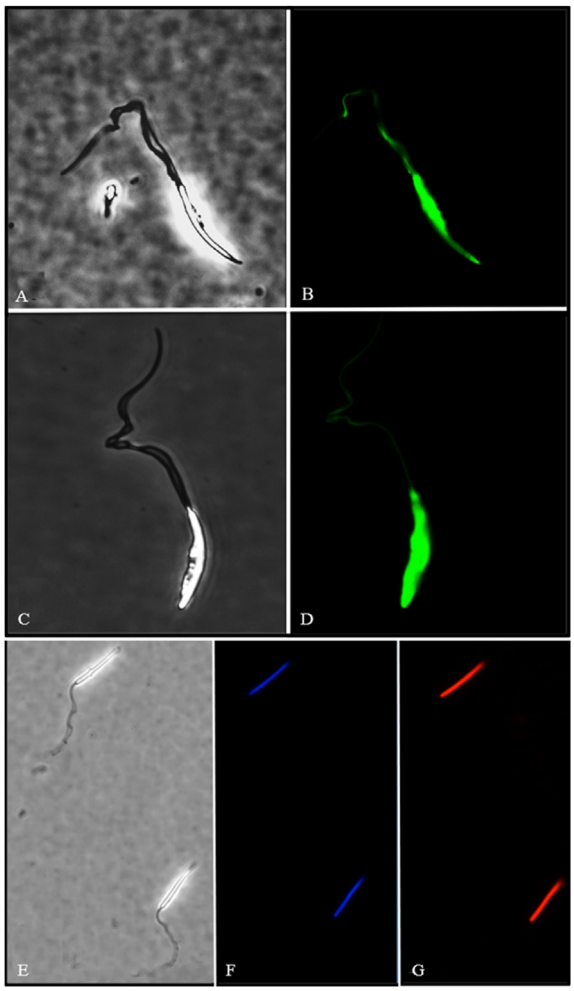

Fig. 7. Panamanian golden frog spermatozoa observed by phase contrast (A, C, and E), after staining the mitochondria with MitoTracker Green (B, D; X 1000 magnification), or the DNA with Hoechst 33342 (F) and Propidium iodide (G; X 400 magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentration present in Amphiplex™ [11]. Metoclopramide might be blocking or attenuating the inhibitory effect of dopamine in *A. zeteki*, or increasing the GnRH-A binding affinity to the receptors, as it has been observed with pimozide and domperidone in fish [39]. This is likely triggering the production of higher concentrations of spermatozoa in the urine, perhaps due to higher luteinizing hormone (LH) release [9,11,15,40,41]. While GnRH-A acts on the pituitary, hCG acts directly on the gonads, since this hormone binds to the same receptors as LH [38,42]. There are several reports on the high species variability of spermiation responses following hCG stimulation [11,15,21,42], with fewer species showing a better response to this hormone than to GnRH-A. This is likely due to the relatively low affinity of hCG to the anuran LH receptor [9,38] or to a possible immune response to this mammalian hormone [43].

Even though all the treatments increased the spermic urine pH, treatment with 4 μg/gbw GnRH-A showed significantly lower pH than 5 IU/gbw hCG and also exhibited lower pH trending towards significance than 2 μg/gbw GnRH-A, 10 IU/gbw hCG and Amphiplex™. Nevertheless, pH did significantly differ among treatments, suggesting that the hormone treatments used in the study increased *A. zeteki*'s spermic urine pH, similar to patterns observed in fish species [44]. We also assessed spermic urine omsolality from all the treatments and found no differences among hormones nor between hormones and controls, suggesting that neither hormone treatment affected the osmolality of the spermic urine in *A. zeteki* nor osmolality had an effect on the lower percentage of motility from 4 μg/gbw GnRH-A. Factors such as pH and ionic composition of the extracellular environment have been shown to play an important role in the regulation of sperm motility in *Xenopus* and ranids [25,27,45]. Even though 4 μg/gbw GnRH-A produced high concentrations of spermatozoa with a good percentage of motility, the lower pH associated with this treatment may account for the lower motility observed in *A. zeteki* spermatozoa, an effect that seems to be counterbalanced by lower GnRH-A concentrations, metoclopramide in the Amphiplex™ treatment and absent in the hCG treatment.

We found that the hormone treatments were equally effective at promoting spermiation in *A. zeteki* year-round with no apparent seasonal effects. If this pattern holds true for females, these technologies could be used to produce offspring year-round in captive settings.

The percentage of motile spermatozoa was higher than 85% for all the hormone stimulation. Frogs treated with GnRH-A exhibited the lowest motility compared to the other treatments that exhibited similar percentage motility during the production peaks. The proportions of motile spermatozoa obtained after stimulation is consistent with reports in other species even without hormonal treatments [9,46]. The slight differences in sperm motility observed in the present study are similar to previous reports in frogs and toads [18,38]. However, the specific mechanism by which hormonal stimulation might affect sperm FPM is not yet understood but others have also observed a dose-dependent relationship between hCG and FPM [18].

A. zeteki sperm has an elongated filiform head with an acrosomal complex on the anterior portion, and a small midpiece at the posterior end of the head. The size and morphology of the spermatozoa were found to be homogeneous in the sperm samples regardless of the hormonal treatments administered. The nucleus has a long, conical structure that occupies most of the head of *A. zeteki* spermatozoa which is similar to the morphology reported for another bufonid and several dendrobatid frogs [47–49]. We also observed the presence of mitochondrial sheaths [13], which are a cytoplasmic droplet-like structure that contains a high concentration of mitochondria, located in the external mid to lower part of the sperm head, sometimes referred as mitochondrial vesicle [50]. The structure is usually present in bufonids and easily damaged by osmotic and temperature changes or handlings [51] making sperm manipulation in *A. zeteki* challenging. The tail is almost twice the length of the head and clearly shows two filaments joined by an undulating membrane, ending in a single filament at the posterior end of the tail. The presence of an undulating membrane and auxiliary fibers in the sperm tail is characteristic of Bufonidae, and other species with aquatic external fertilization modes [23,47,52]. Spermatozoa of *A. spumarius* have an average head size of 19 μm and a tail length of 40 μm [47]. Therefore, the head of *A. zeteki* sperm is slightly bigger while the tail length is similar to *A. spumarius*. To our knowledge, this is the first detailed study on *A. zeteki* sperm morphology. On average about 75% of the cells were normal and did not differ between hormone treatments. Testicular sperm had a lower percentage of normal tails than excreted sperm probably because testicular sperm samples include a mixture of mature and immature cells and tails may have been damaged during maceration.

The proportion of cells with DNA fragmentation was not different after the various stimulation protocols and was similar to the proportions observed in the testicular spermatozoa. Studies on sperm DNA fragmentation report lower percentages with a cutoff for infertile men at 20% [28]. While there is no established fertility

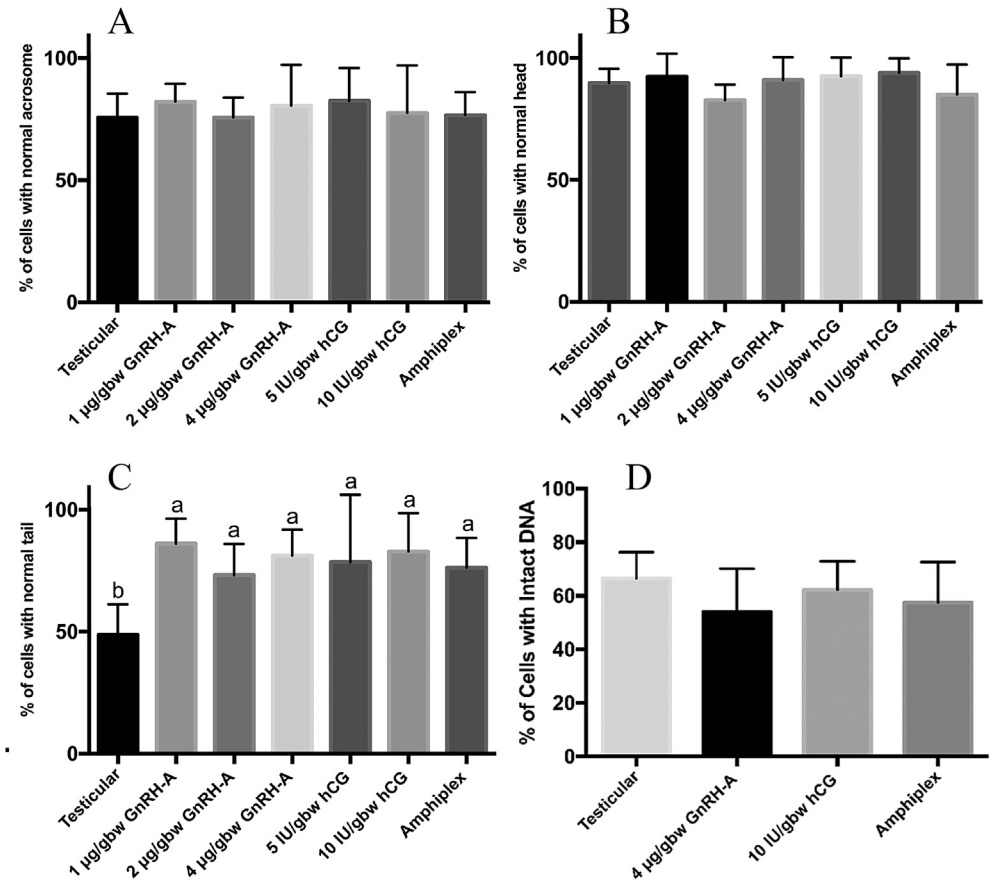

Fig. 8. Percentage of normal spermatozoa by morphological feature after each hormone treatment (n = 27 males) and in control testicular spermatozoa (n = 6 males) and percentage of spermatozoa with intact DNA after best hormone treatments. Normal acrosome (A), normal head (B), normal tail (C), intact DNA (D). Values are expressed as mean ± SD. Bars with different letters are statistically different ($P < 0.05$).

threshold for anurans, 40% of sperm with fragmented DNA seems high in this species as observed in *Rana temporaria* [53]. Breakage in the nuclear chromatin can be related to the following factors: 1) chromatin is highly compacted inside the sperm nucleus where topoisomerases might relieve torsional stress by inducing nicks in the DNA [28]; 2) amphibian sperm activates after a drop in the osmolality of the extracellular environment, which causes an osmotic shock that might induce DNA fragmentation [25,29,54]; 3) sperm manipulation can increase the DNA susceptibility for damage; 4) the TUNEL assay does not discriminate between viable or dead cells, suggesting that the overall DNA fragmentation value could include non-viable cells [28]. It is possible that after fertilization oocyte-repairing mechanisms may be activated [55] to reduce the impact of sperm DNA damage on embryo development.

In conclusion, our study provides a systematic description of *A. zeteki* sperm characteristics as a reference for other species, and as a baseline to evaluate potential future changes within captive lines. We also describe an optimized, safe and repeatable sperm collection protocol for *A. zeteki*. This is a critical first step in developing reproductive technologies and sperm cryopreservation to manage captive populations.

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